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(54) Title: INHIBITORS OF DIPEPTIDYL-AMINOPEPTIDASE TYPE IV

(57) Abstract

The invention is drawn to potent inhibitors of the enzymatic activity of DP-IV wherein the inhibitors are amino boronic acid analogs of protein.

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INHIBITORS OF DIPEPTIDYL-AMINOPEPTIDASE TYPE IV

Background of the Invention

This invention relates to inhibitors of the amino
5   peptidase activity of dipeptidyl peptidase type IV (DP-
IV).

DP-IV is a postproline cleaving enzyme with a
specificity for removing Xaa-Pro (where Xaa represents
any amino acid) dipeptides from the amino terminus of
10   polypeptides. DP-IV will also remove Xaa-Ala dipeptides
from amino termini, albeit less efficiently. DP-IV is
present in many mammalian cells and tissues, for example,
renal tubule cells, intestinal epithelium, and blood
plasma. It is also present on the surface of CD4+ and
15   some CD8+ T-cells. It is thought to be involved in the
regulation of the immune response; occurrence of DP-IV on
a cell surface is associated with the ability of cells to
produce interleukin-2 (IL-2). DP-IV is also referred to
as dipeptidyl-peptide hydrolase DAP-IV or DPP-IV; it is
20   assigned EC number 3.4.14.5.

Three different inhibitors of DP-IV are known.
One of these is a suicide inhibitor: N-Ala-Pro-O-
(nitrobenzyl-) hydroxylamine. (The standard three letter
amino acid codes are used in this application; O
25   represents oxygen.) Another is a competitive inhibitor:
e-(4-nitro) benzoxycarbonyl-Lys-Pro. The third is a
polyclonal rabbit anti-porcine kidney DP-IV
immunoglobulin.

Summary of the Invention

30   The enzymatic activity of DP-IV involves cleaving
of a dipeptide from the free amino terminus of a
polypeptide. DP-IV has a preference for cleaving after a
proline, i.e., a proline in the penultimate position from
the amino terminus. A free amino terminus is required;
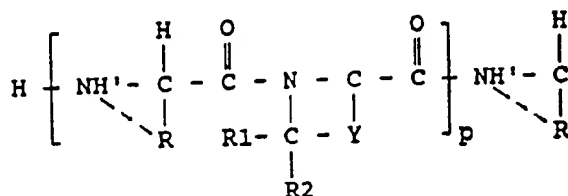35   thus, DP-IV is a postproline cleaving enzyme with a

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specificity for removing an N-terminal Xaa-Pro dipeptide
from a polypeptide (where Xaa can be any amino acid,
including proline). DP-IV also will remove a Xaa'-Ala
dipeptide from an amino terminus of a polypeptide when
5   Xaa' is an amino acid with a bulky side group, e.g.,
tyrosine.

This invention concerns provision of potent
inhibitors of the enzymatic activity of DP-IV.
Generally, an α-amino boronic acid analog of proline
10 (boroPro is used to designate one such analog which has
the carboxyl group of proline replaced with a $B(OH)_2$
group, where $(OH)_2$ represents two hydroxyl groups and B
represents boron) is bonded to an amino acid to form a
dipeptide with boroPro as the carboxy terminal residue.
15 These dipeptide prolyl-boronic acids are potent and
highly specific inhibitors of DP-IV, with K_i values in
the nanomolar range.

Dipeptides having the boroPro moiety are
relatively unstable; thus, we have designed inhibitors
20 having at least two other amino acid residues.
Generally, the structure of these inhibitors is X-Pro-Y-
boroPro where X and Y are chosen from any amino acid
residue (including proline). This tetrapeptide may be
lengthened at its amino-terminus by addition of one or
25 more dipeptides, each dipeptide having the general
formula Z-Pro or Z-ala, where each Z independently is any
amino acid residue (including proline). This general
structure is defined in more detail below. These
inhibitors function as inhibitors of DP-IV because each
30 dipeptide portion is a substrate for DP-IV and the final
product of the reaction of such an inhibitor with DP-IV
is the dipeptide inhibitor Y-boroPro. The amino terminus
of these inhibitors must not be blocked or they lose
their inhibitory capacity for DP-IV, since DP-IV cannot
35 cleave a dipeptide from a blocked N-terminal polypeptide.

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Thus, in a first aspect, the invention features an
inhibitory compound having the structure: Group I -
Group II. Group I has the structure:

$$
H \left[\mathrm{NH'} - \underset{\underset{R}{\diagdown}}{\overset{\overset{H}{|}}{C}} - \overset{\overset{O}{||}}{C} - N - \underset{\underset{\underset{R2}{|}}{R1 - C - Y}}{C} - \overset{\overset{O}{||}}{C} \right]_{p} \mathrm{NH'} - \underset{\underset{R}{\diagdown}}{\overset{\overset{H}{|}}{C}}
$$

where H represents a hydrogen; C represents a carbon; O
represents an oxygen; N represents a nitrogen; each R,
independently, is chosen from the group consisting of the
R groups of an amino acid, including proline; each broken
line, independently, represents a bond to an H or a bond
to one R group, and each H' represents that bond or a
hydrogen; and p is an integer between 0 and 4 inclusive.
Alternatively, Group I has the structure:

$$
G1 \left[\underset{\underset{G3}{|}}{\overset{\overset{G2}{|}}{C}} \right]_{n}
$$
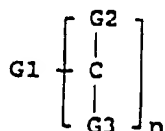
where n is between 0 and 3 inclusive, each G2 and G3
independently is H or C1 - 3 (one to three carbon atoms)
alkyl, G1 is NH3 (H3 represents three hydrogens),

$$
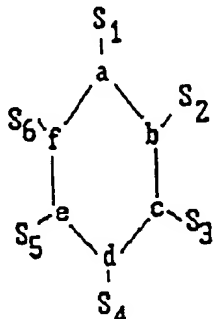\underset{\underset{NH2}{||}}{NH - C - NH2}
$$
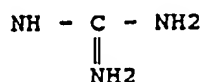
(H2 represents two hydrogens), or

NG4, where G4 is $\underset{\underset{G6}{|}}{C - G5}$
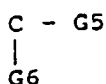
where G5 and G6 can be NH, H, or C1 - 3 alkyl or alkenyl
with one or more carbons substituted with a nitrogen. G1

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bears a charged, and G1 and Group II do not form a
covalently bonded ring structure at pH 7.0. Group I may
also have the structure:

$$
\begin{array}{c}
S_1 \\
| \\
a \\
S_6\diagdown f \diagup \quad \diagdown b \diagup S_2 \\
| \qquad | \\
S_5\diagup e \diagdown \quad \diagup c \diagdown S_3 \\
\diagdown d \diagup \\
| \\
S_4
\end{array}
$$

where one or two of the a, b, c, d, e, and f group is N,
5 and the rest are C, and each S1 - S6 independently is H
or C1 - C3 alkyl. Group I may also include a five
membered unsaturated ring having two nitrogen atoms,
e.g., an imidazole ring. Group II has the structure:

$$
\begin{array}{c}
\qquad O \qquad\; H \\
\qquad \| \qquad\; | \\
-\quad C - N - C - T \\
\qquad\; | \qquad | \\
R1- C - Y \\
\qquad | \\
R2
\end{array}
$$
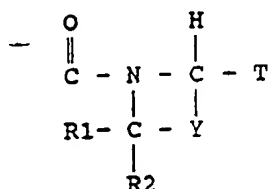
where T is a group of the formula:

10
$$
\begin{array}{c}
D2 \\
| \\
- B - D1,
\end{array}
$$
where each D1 and D2, independently, is a
hydroxyl group or a group which is capable of being
hydrolysed to a hydroxyl group in aqueous solution at
15 physiological pH; a group of the formula:

$$
\begin{array}{c}
- C \quad - CF - G, \\
\| \qquad | \\
O \qquad F
\end{array}
$$
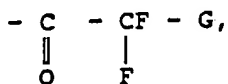
- 5 -

where G is either H, fluorine (F) or an alkyl group
containing 1 to 20 carbon atoms and optional heteroatoms
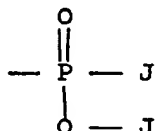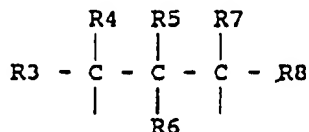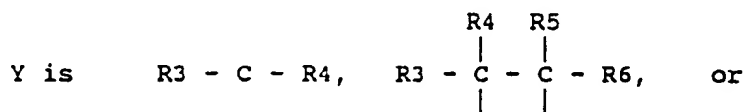which can be N, S (sulfur), or O; or a phosphonate group
of the formula:

$$
\begin{array}{c}
O \\
\parallel \\
- \; P \; - \; J \\
\mid \\
O \; - \; J
\end{array}
$$

where each J, independently, is O-alkyl, N-alkyl, or
alkyl. Each O-alkyl, N-alkyl or alkyl includes 1 - 20
carbon atoms and, optionally, heteroatoms which can be N,
S, or O. T is generally able to form a complex with the
catalytic site of a DP-IV.

$$
\text{Y is} \qquad R3 - \overset{\displaystyle }{\underset{\displaystyle }{C}} - R4, \qquad
R3 - \overset{\displaystyle R4}{\underset{\displaystyle }{C}} - \overset{\displaystyle R5}{\underset{\displaystyle }{C}} - R6, \qquad \text{or}
$$

$$
R3 - \overset{\displaystyle R4}{\underset{\displaystyle }{C}} - \overset{\displaystyle R5}{\underset{\displaystyle R6}{C}} - \overset{\displaystyle R7}{\underset{\displaystyle }{C}} - R8
$$

and each R1, R2, R3, R4, R5, R6, R7, and R8, separately
is a group which does not significantly interfere with
site specific recognition of the inhibitory compound by
DP-IV, and allows a complex to be formed with DP-IV.
In preferred embodiments, T is a boronate group, a
phosphonate group or a trifluoroalkyl ketone group; each
R1-R8 is H; each R1 and R2 is H, and each Y is the CH_2-
CH_2; each R is independently chosen from the R group of
proline and alanine; the ihibitory compound has a binding
or dissociation constant to DP-IV of at least $10^{-9}M$, $10^{-8}M$
or even $10^{-7}M$; the inhibitory compound is admixed with a
pharmaceutically acceptable carrier substance; and each

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D1 and D2 is, independently, F, or D1 and D2 together are
a ring containing 1 to 20 carbon atoms, and optionally
heteroatoms which can be N, S, or O.

In a second aspect, the invention features a
5 method for inhibiting the enzymatic activity of DP-IV in
a mammal. The method includes administering to the
mammal an effective amount of an inhibitory compound
described above. Most preferably, the amount of compound
administered is between 1 and 500 mg/kilogram of animal
10 treated/day.

In a third aspect, the invention features an
inhibitor of DP-IV, having the structure:

$$
\left[A - N \begin{array}{c} H \\ | \\ \end{array} C \begin{array}{c} | \\ CH_2 \\ \diagdown CH_2 \diagup \end{array} \overset{O}{\overset{||}{C}} \right]_m A' - N \begin{array}{c} H \\ | \\ CH_2 \end{array} C \begin{array}{c} | \\ CH_2 \\ \diagdown CH_2 \diagup \end{array} B \diagup^{X^1} \diagdown_{X^2}
$$
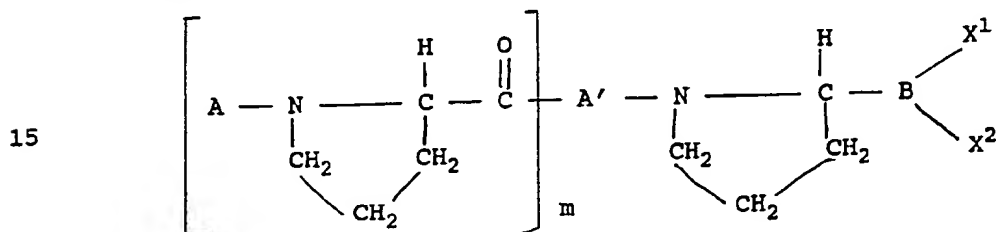
wherein m is an integer between 0 and 10, inclusive; A
and A' are L-amino acid residues (for glycine there is no
20 such distinction) such that the A in each repeating
bracketed unit can be a different amino acid residue; the
C bonded to B is in the L-configuration; the bonds
between A and N, A' and C, and between A' and N are
peptide bonds; and each X^1 and X^2 is, independently, a
25 hydroxyl group or a group capable of being hydrolysed to
a hydroxyl group at physiological pH. By "the C bonded
to B is in the L-configuration" is meant that the
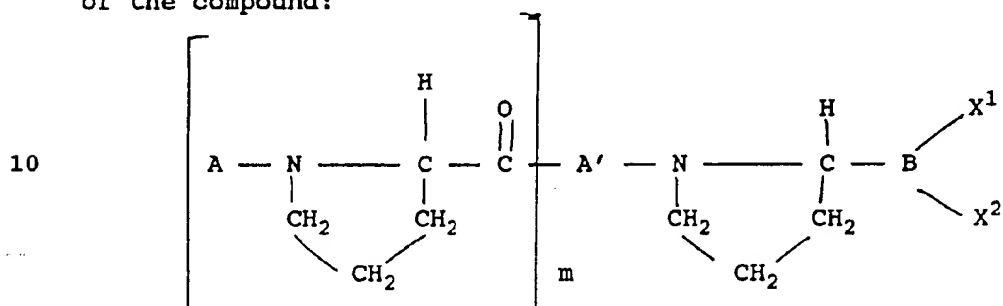absolute configuration of the C is like that of an L-
amino acid.

30 Thus the $B \diagup^{X_1} \diagdown_{X_2}$ group has the same relationship to the C

as the -COOH group of an L-amino acid has to its α
carbon. In various preferred embodiments, A and A' are

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independently proline or alanine residues; m is 0; X^1 and X^2 are hydroxyl groups; the inhibitor is L-Ala-L-boroPro; and the inhibitor is L-Pro-L-boroPro.

In a fourth aspect, the invention features a method for inhibiting DP-IV in a mammal. The method includes administering to the mammal an effective amount of the compound:

$$\left[\begin{array}{c} \underset{CH_2}{\underset{|}{\overset{H}{\underset{|}{N}}}} \\ A - N \rule{2em}{0.4pt} \underset{\underset{\diagdown \ CH_2 \diagup}{\underset{|}{CH_2}}}{C} - \overset{\overset{O}{\|}}{C} \end{array} \right]_m A' - \underset{\underset{\diagdown \ CH_2 \diagup}{\underset{|}{CH_2}}}{\overset{\overset{H}{\underset{|}{N}}}{N}} \rule{2em}{0.4pt} \underset{\underset{|}{CH_2}}{C} - B \begin{array}{c} \diagup X^1 \\ \diagdown X^2 \end{array}$$

described above. In a preferred embodiment, the amount is 1 mg/kg of the mammal per day to 500 mg/kg of the mammal per day.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments, and from the claims.

Description of the Preferred Embodiments

The drawings will first be briefly described.

Drawings

Fig. 1 is a diagrammatic representation of the synthesis of a boro proline compound; and

Fig. 2 is a diagrammatic representation of several embodiments of the invention.

Structure

The inhibitory compounds of the invention have the general structure recited in the Summary of the Invention above. Examples of preferred structures are those referred to as preferred embodiments above.

The structure of the preferred inhibitory compounds is such that at least a portion of the amino acid sequence near the cleavage site of a DP-IV substrate

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is duplicated, or nearly duplicated. This duplication is
in part responsible for the ability of the inhibitory
compounds to inhibit DP-IV, by a mechanism thought to
involve competitive inhibition between a DP-IV inhibitory
5   compound or a DP-IV cleavage product of the inhibitory
compound, and the actual DP-IV substrate.

The choice of amino acid sequence affects the
inhibitory activity of the inhibitory compound, and its
specificity. Peptide fragments can be synthesized and
10 then tested to determine their efficacy as inhibitors,
using standard techniques. Specificity is determined in
a similar fashion, by testing the inhibitory effect of a
particular inhibitory compound on the enzyme activity.
The inhibitory compounds preferably inhibit the enzymatic
15 activity of DP-IV and do not inhibit enzymes necessary
for normal cell functions.

The inhibitory compounds include a group (T) which
causes the inhibitory compound to complex with DP-IV, not
only in a competitive fashion, but in a chemically
20 reactive manner to form a strong bond between the
inhibitory compound and DP-IV. This group thus acts to
bind the inhibitory compound to DP-IV, and increases the
inhibitory binding constant (K_i) of the inhibitory
compound. Examples of such groups include boronates,
25 fluoroalkyl ketones and phosphoramidates (of the formulae
given in the Summary above). These groups are covalently
bonded to the prolyl residue of the compound, as in the
above formula.

The proline or proline analog, represented by

30
$$
\begin{array}{c}
- \text{N} - \text{C} - \\
| \qquad | \\
\text{R1} - \text{C} - \text{Y} \\
| \\
\text{R2}
\end{array}
$$
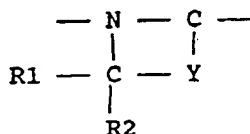
above, is chosen so that it mimics the structure of
proline recognized by the active site of DP-IV. It can
35 be modified by providing R1 and R2 groups which do not

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interfere significantly with this recognition, and thus
do not significantly affect the K_i of the compound.
Thus, one or more hydroxyl groups can be substituted to
form hydroxy-proline, and methyl or sugar moieties may be
5   linked to these groups. One skilled in the art will
recognize that these groups are not critical in this
invention and that a large choice of substituents are
acceptable for R1 and R2. In part, the requirement that
the above described proline analog mimics the structure
10   of proline recognized by the active site of DP-IV means
that the C bonded to N and Y has the same stereochemistry
as the alpha carbon of L-proline.
Synthesis of BoroProline
        Referring to Fig. 1, the starting compound I is
15   prepared essentially by the procedure of Matteson et al.
(*Organometallics* 3:1284, 1984), except that a pinacol
ester is substituted for the pinanediol ester. Similar
compounds such as boropipecolic acid and 2-azetodine
boronic acid can be prepared by making the appropriate
20   selection of starting material to yield the pentyl and
propyl analogs of compound I. Further, Cl can be
substituted for Br in the formula, and other diol
protecting groups can be substituted for pinacol in the
formula, e.g., 2, 3-butanediol and alpha-pinanediol.
25   Compound II is prepared by reacting compound I
with $[(CH_3)O_3Si]_2N\text{-}Li+$. In this reaction
hexamethyldisilazane is dissolved in tetrahydrofuran and
an equivalent of n-butyllithium added at -78°C. After
warming to room temperature (20°C) and cooling to -78°C,
30   an equivalent of compound I is added in tetrahydrofuran.
The mixture is allowed to slowly come to room temperature
and to stir overnight. The alpha-bis[trimethylsilane]-
protected amine is isolated by evaporating solvent and
adding hexane under anhydrous conditions. Insoluble

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residue is removed by filtration under a nitrogen
blanket, yielding a hexane solution of compound II.

Compound III, the N-trimethysilyl protected form
of boroProline is obtained by the thermal cyclization of
5   compound II during the distillation process in which
compound II is heated to 100-150° and distillate is
collected which boils 66-62°C at 0.06-0.10 mm pressure.

Compound IV, boroProline-pinacol hydrogen
chloride, is obtained by treatment of compound III with
10   HCl:dioxane. Excess HCl and by-products are removed by
trituration with ether. The final product is obtained in
a high degree of purity by recrystallization from ethyl
acetate.

The boroProline esters can also be obtained by
15   treatment of the reaction mixture obtained in the
preparation of compound II with anhydrous acid to yield
1-amino-4-bromobutyl boronate pinacol as a salt.
Cyclization occurs after neutralizing the salt with base
and heating the reaction.

20   Preparation of boroProline-pinacol

The intermediate, 4-Bromo-1-chlorobutyl boronate
pinacol, was prepared by the method in Matteson et al.
(Organometallics 3:1284, 1984) except that conditions
were modified for large scale preparations and pinacol
25   was substituted for the pinanediol protecting group.

3-bromopropyl boronate pinacol was prepared by
hydrogenboronation of allyl bromide (173 ml, 2.00 moles)
with catechol borane (240 ml, 2.00 moles). Catechol
borane was added to allyl bromide and the reaction heated
30   for 4 hours at 100°C under a nitrogen atmosphere. The
product, 3-bromopropyl boronate catechol (bp 95-102°C,
0.25 mm), was isolated in a yield of 49% by distillation.
The catechol ester (124 g, 0.52 moles) was
transesterified with pinacol (61.5 g, 0.52 moles) by
35   mixing the component in 50 ml of THF and allowing them to

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stir for 0.5 hours at 0°C and 0.5 hours at room
temperature. Solvent was removed by evaporation and 250
ml of hexane added. Catechol was removed as a
crystalline solid. Quantitative removal was achieved by
5   successive dilution to 500 ml and to 1000 ml with hexane
and removing crystals at each dilution. Hexane was
evaporated and the product distilled to yield 177 g (bp
60 - 64°C, 0.35 mm).
        4-Bromo-1-chlorobutyl boronate pinacol was
10   prepared by homologation of the corresponding propyl
boronate. Methylene chloride (50.54 ml, 0.713 moles) was
dissolved in 500 ml of THF, 1.54 N n-butyllithium in
hexane (480 ml, 0.780 moles) was slowly added at -100°C.
3-Bromopropyl boronate pinacol (178 g, 0.713 moles) was
15   dissolved in 500 ml of THG, cooled to the freezing point
of the solution, and added to the reaction mixture. Zinc
chloride (54.4 g, 0.392 moles) was dissolved in 250 ml of
THG, cooled to 0°C, and added to the reaction mixture in
several portions. The reaction was allowed to slowly
20   warm to room temperature and to stir overnight. Solvent
was evaporated and the residue dissolved in hexane (1
liter) and washed with water (1 liter). Insoluble
material was discarded. After drying over anhydrous
magnesium sulfate and filtering, solvent was evaporated.
25   The product was distilled to yield 147 g (bp 110 - 112°C,
0.200 mm).
        N-Trimethylsilyl-boroProline pinacol was prepared
first by dissolving hexamethyldisilizane (20.0 g, 80.0
mmoles) in 30 ml of THF, cooling the solution to -78°C,
30   and adding 1.62 N n-butyllithium in hexane (49.4 ml, 80.0
mmoles). The solution was allowed to slowly warm to room
temperature. It was recooled to -78°C and 4-bromo-1-
chlorobutyl boronate pinacol (23.9 g, 80.0 mmoles) added
in 20 ml of THF. The mixture was allowed to slowly warm
35   to room temperature and to stir overnight. Solvent was

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removed by evaporation and dry hexane (400 ml) added to
yield a precipitant which was removed by filtration under
a nitrogen atmosphere. The filtrate was evaporated and
the residue distilled, yielding 19.4 g of the desired
5   product (bp 60 - 62°C, 0.1 - 0.06 mm).

        H-boroProline-pinacol.HCl (boroProline-
pinacol.HCl) was prepared by cooling N-trimethylsilyl-
boroProline pinacol (16.0 g, 61.7 mmoles) to -78°C and
adding 4 N HCL:dioxane 46 ml, 185 mmoles). The mixture
10 was stirred 30 minutes at -78°C and 1 hour at room
temperature. Solvent was evaporated and the residue
triturated with ether to yield a solid. The crude
product was dissolved in chloroform and insoluble
material removed by filtration. The solution was
15 evaporated and the product crystallized from ethyl
acetate to yield 11.1 g of the desired product (mp 156.5 -
    157°C).

Synthesis of boroProline Peptides

        General methods of coupling of N-protected
20 peptides and amino acids with suitable side-chain
protecting groups to H-boroProline-pinacol are
applicable. When needed, side-chain protecting and N-
terminal protecting groups can be removed by treatment
with anhydrous HCl, HBr, trifluoroacetic acid, or by
25 catalytic hydrogenation. These procedures are known to
those skilled in the art of peptide synthesis.

        The mixed anhydride procedure of Anderson et al.
(J. Am. Chem. Soc. 89:5012, 1984) is preferred for
peptide coupling. Referring again to Fig. 1, the mixed
30 anhydride of an N-protected amino acid or a peptide is
prepared by dissolving the peptide in tetrahydrofuran and
adding one equivalent of N-methylmorpholine. The
solution is cooled to -20°C and an equivalent of isobutyl
chloroformate is added. After 5 minutes, this mixture
35 and one equivalent of triethylamine (or other sterically

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hindered base) are added to a solution of H-boroPro-
pinacol dissolved in either cold chloroform of
tetrahydrofuran.

The reaction mixture is routinely stirred for one
5   hour at -20°C and 1 to 2 hours at room temperature
(20°C). Solvent is removed by evaporation, and the
residue is dissolved in ethyl acetate. The organic
solution is washed with 0.20N hydrochloric acid, 5%
aqueous sodium bicarbonate, and saturated aqueous sodium
10 chloride. The organic phase is dried over anhydrous
sodium sulfate, filtered, and evaporated. Products are
purified by either silica gel chromatography or gel
permeation chromatography using Sephadex™ LH-20 and
methanol as a solvent.
15   Previous studies have shown that the pinacol
protecting group can be removed *in situ* by preincubation
in phosphate buffer prior to running biological
experiments (Kettner et al., *J. Biol. Chem.* 259:15106,
1984). Several other methods are also applicable for
20 removing pinacol groups from peptides, including
boroProline, and characterizing the final product.
First, the peptide can be treated with diethanolamine to
yield the corresponding diethanolamine boronic acid
ester, which can be readily hydrolyzed by treatment with
25 aqueous acid or a sulfonic acid substituted polystyrene
resin as described in Kettner et al. (*supra*). Both
pinacol and pinanediol protecting groups can be removed
by treating with BC13 in methylene chloride as described
by Kinder et al. (*J. Med. Chem.* 28:1917). Finally, the
30 free boronic acid can be converted to the difluoroboron
derivative (-BF2) by treatment with aqueous HF as
described by Kinder et al. (*supra*).

Similarly, different ester groups can be
introduced by reacting the free boronic acid with various

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di-hydroxy compounds (for example, those containing
heteroatoms such as S or N) in an inert solvent.

Preparation of H-Ala-boroPro

Boc-Ala-boroPro was prepared by mixed anhydride
5   coupling of the N-Boc-protected alanine and H-boroPro
prepared as described above. H-Ala-boroPro (Ala-boroPro)
was prepared by removal of the Boc protecting group at
0°C in 3.5 molar excess of 4N HCl-dioxane. The coupling
and deblocking reactions were performed by standard
10   chemical reaction. Ala-boroPro has a K_i for DP-IV of in
the nanomolar range. Boc-blocked Ala-boroPro has no
affinity for DP-IV.

The two diastereomers of Ala-boroPro-pinacol,
L-Ala-D-boroPro-pinacol and L-Ala-L-boroPro-pinacol, can
15   be partially separated by silica gel chromatography with
20% methanol in ethyl acetate as eluant. The early
fraction appears by NMR analysis to be 95% enriched in
one isomer. Because this fraction has more inhibits DP-
IV to a greater extent than later fractions (at equal
20   concentrations) it is probably enriched in the L-boroPro
(L-Ala-L-boroPro-pinacol) isomer.

One significant drawback with H-Ala-boroPro as an
inhibitor for DP-IV is that is decomposes in aqueous
solution at neutral pH and room temperature (20 - 25°C)
25   with a half-life of around 0.5 hour. Many dipeptide
derivatives with a free N terminal amino group and a
functional group (such as a difluoromethyl ketone) on the
C-terminus are similarly unstable due to intramolecular
reaction. A six member ring is formed between the amino
30   and C-terminal functional groups and undergoes subsequent
further reaction, such as hydrolysis. DP-IV bound
inhibitor is more stable, consistent with the hypothesis
that decomposition is due to an intramolecular reaction.

H-Pro-boroPro is more stable than H-Ala-boroPro.
35   The K_i of H-Pro-boroPro for DP-IV is about 1×10^{-8}M, and

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it decomposes in aqueous solution at room temperature
(20-25°C) with a half life of about 1.5 hours. Although
the affinity of H-Pro-boroPro is about 10-fold less than
that of H-Ala-boroPro, the increased stability is
5   advantageous.

        Because of the relatively short half life of the
above dipeptides inhibitory compounds of the invention
are formed as tetrapeptides or longer peptides as shown
in the general formula above. These inhibitory compounds
10   are substrates for DP-IV yielding the dipeptide inhibitor
A'-boroPro. These polypeptide boronic acids are
generally stable and can be administered by any standard
procedure to act as a substrate for DP-IV and then as a
source of a potent DP-IV inhibitor. The advantages of
15   such molecules is that inhibitor is released only in the
vicinity of active DP-IV. These polypeptide boronic
acids can be made by the method of mixed anhydride
coupling by one of ordinary skill in the art, e.g.,
Mattason (*Organometallics* 3:1284, 1984).
20   Assays for DP-IV Inhibition

        The following are examples of systems by which the
inhibitory activity of the above described inhibitory
compounds can be tested on DP-IV. As an example H-Ala-
boroPro is used to test each of these systems.
25   Inhibitory compounds can be tested by simply substituting
them for H-Ala-boroPro.

        DP-IV is purified from pig kidney cortex by the
method of Barth et al. (*Acta Biol. Med. Germ.* 32:157,
1974) and Wolf et al. (*Acta Biol. Med. Germ.* 37:409,
30   1978) and from human placenta by the method of Puschel et
al. (*Eur. J. Biochem.* 126:359, 1982). H-Ala-boroPro
inhibits both enzymes with a K_i in the nanomolar range.
Human Peripheral blood Mononuclear Cells

        H-Ala-boroPro was tested for its influence on PHA-
35   induced proliferation of human peripheral blood

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mononuclear cells. Human peripheral blood mononuclear
cells were obtained from healthy human donors by Ficoll-
Hypaque density gradient centrifugation. The cells are
washed three times in RPMI 1640 medium and resuspended to
5   a concentration of a 1×10^6 in RPMI. 10% human serum
was used as necessary.

The proliferative response of lymphocytes was
measured using 3H-Thymidine incorporation. 5×10^3 MNC
cells (Ford in *Handbook of Experimental Immunology*, Weir,
10   ed., Blackwell Scientific Publications, Oxford, 1978)
were distributed into wells of round-bottom microtiter
plates and incubated in the presence or absence of
various dilutions of antigen, mitogen, lymphokine or
other agent of interest. Cells were cultured in an
15   atmosphere of 5% CO_2 in air for 72 hours after which ^{3}H-
Thymidine (0.5 μCl/well; 2.0 Ci/mM, New England Nuclear)
was added 6 hours before termination of culture. The
cells were harvested with a multiple automatic harvester,
and ^{3}H-thymidine incorporation assessed by liquid
20   scintillation counting. ^{3}H thymidine incorporation was
determined relative to control values in the absence of
inhibitor. Inhibitor was added to give a final
concentration of 1×10^{-4}M, but lower concentrations can
be used.

25   HIV gene replication

We examined the effect of H-Ala-boroPro on HIV-1
replication *in vitro*. The rational for these experiments
comes from the reported connection between T-cell
activation, IL-2 production, and HIV replication and
30   expression of HIV proteins. For example, inductive
signals associated with HIV replication include mitogens,
antigens, lymphokines, and transcriptions factors such as
NF-κB, all of which have been shown to be associated with
induction of IL-2 production, T-cell activation, or both.

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Cell lines used in the present studies include
A3.5 cells (a monocyte cell line which is CD4+, HLA-DR+,
and CD3-) and peripheral blood mononuclear cells (PBMC).
The A3.5 cells grow continuously in culture without
5   exogenous growth factors. PBMC cells require IL-2 for
propagation *in vitro*. Cells were infected with HIV-1-
IIIB at a multiplicity of infection (moi) of 5 x 10^{-4}
tissue culture infectious dose 50 (TCID50)/cell for both
the A3.5 cells and the PMBC cells. Dilutions of
10 inhibitor were made in RPMI-1640 and subsequently passed
through a 0.22 um filter. At the start of each
experiment, 1 x 10^6 cells/well, in 24-well plates, were
infected with HIV-1-IIIB at the moi indicated above.
Inhibitor was added simultaneously at the appropriate
15 dilutions. All cultures were maintained at 5% CO_2 and
37°C in RPMI-1640 supplemented with penicillin,
streptomycin, L-glutamine, hepes buffer, and 20% heat-
inactivated fetal calf serum. Cell counts and viability
were determined by trypan blue exclusion. Culture
20 supernatants were harvested and assayed for HIV-1 p24
antigen by ELISA (NEN-DuPont, Boston, MA). Fresh media
and inhibitor were added on each day. For PBMC cultures,
cells were collected from HIV-1 seronegative donors and
stimulated with PHA-P (Difco, Detroit, MI; 10 μg/ml) and
25 10% IL-2 (Electronnucleonics, Silver Spring, MD) 3 days
prior to infection with HIV-1. PBMC cultures for all
experiments included uninfected and infected cells
without inhibitor, uninfected cells with inhibitor at the
various concentrations, and infected cells in the
30 presence of 1 μm zidovudine (azidothymidine, AZT).

With A3.5 H-Ala-boroPro suppresses HIV below
detectable levels in a manner similar to the anti-HIV
effect of AZT at 1 μm. Similar results were observed
with the PBMC cells. Thus, inhibitors of this invention
35 have an anti-HIV effect. Cell viability assays show that

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these inhibitors are not cytotoxic even at relatively
high concentration (10^{-3}M for A3.5 cells).
Determination of DP-IV Activities in Biological Samples
          The ability to determine DP-IV activities
5   associated with cells and tissues is highly desirable.
For example, it will permit correlations to be made
between level of inhibition of DP-IV and the magnitude of
the observed biological affect, e.g., on cell
proliferation, and IL-2 production. Such correlation is
10   helpful in establishing whether or not the biological
effect is due to inhibition of DP-IV. We have found that
such determinations can be reproducibly and reliably made
using the readily available chromogenic substrates for
DP-IV: X-Pro-p-nitroanilides and X-Pro-7-amino-4-
15   trifluoromethyl coumarins (AFC). The AFC substrates are
fluorescent and thus provide greater sensitivity. DP-IV
activity is measured as release of p-nitroanilide
spectrophotometrically at 410 nM, or using X-Pro-AFC
derivatives and measuring fluorescence at 505 nM.
20   Reduction in activity in the presence of inhibitor
provides an easy test for inhibitory activity.
Effect of Inhibitor Stereochemistry on DP-IV Inhibition
          Experiments described below demonstrate that Ala-
boroPro and Pro-boroPro are potent inhibitors of DP-IV
25   with K_i values in the nanomolar range. In addition, the
L,L form of Pro-boroPro is shown to be a far more potent
inhibitor of DP-IV than the L,D form of Pro-boroPro.
          The activity of DP-IV, isolated from porcine
kidneys by the method of Wolf et al. (ACTA Bio. Mes. Ger.
30   37:409, 1972), was measured using Ala-Pro-p-nitroanilide
as a substrate. Briefly, a reaction containing 50 μmol
sodium Hepes (pH7.8), 10 μmol Ala-Pro-p-nitroanilide, 6
milliunits of DP-IV, and 2% (vol/vol) dimethylformamide
in a total volume of 1.0 ml. The reaction was initiated

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by the addition of enzyme and reaction rates were
measured at 25°C.

The rates of DP-IV-catalyzed hydrolysis of Ala-
Pro-*p*-nitroanilide were determined at 3 to 5 different
5   concentration of Ala-boroPro Pro-boroPro, boroPro and *N*-
Boc-Ala-boroPro. In some cases, the initial reactions
rates were not linear. The rates became linear after 10
min; this linear portion can be duplicated by
preincubating enzyme with inhibitor for 10 min before
10   adding substrate. Table 1 presents the results of K_i
measurements made over the linear range.

Table 1: Inhibition constants of some inhibitors of DP-
IV

Inhibitor	K_i nM
N-Boc-Al-boroPro	>1,000,000*
BoroPro	110,000
Ala-boroPro	2
Pro-boroPro	3

20   *No inhibition detected.

Ala-boroPro was a potent inhibitor of DP-IV,
having a K_i value of 2×10^{-9}M (Table 1). Blocking the N
terminus of this inhibitor (e.g., *N*-Boc-Ala-boroPro;
25   Table 1) abolished inhibition, demonstrating that a free,
positively charged amino group is likely essential for
enzyme recognition and binding. The K_i of 3×10^{-9} M for
Pro-boroPro demonstrates that DP-IV tolerates an imino
group in place of the amino functional group on the N
30   terminus as well as the substitution of a proline side
chain in place of the alanine methyl group. This shows
that the S2 specificity subsite is not highly
restrictive. Although DP-IV will accept nearly any amino
acid at the N terminus, interactions between this amino

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acid and the enzyme are critical for binding. This is
illustrated by the 10^5-10^6 decrease in affinity on going
from Ala-boroPro or Pro-boroPro to boroPro itself (Table
1).

5   The inhibition experiments presented in Table 1
were carried out on DP-IV isolated from pig kidneys.
Pro-boroPro and Ala-boroPro inhibit DP-IV from human
placenta equally well.

        The Ala-boroPro and Pro-boroPro used in the
10   experiments described above were raecemic mixtures in
which the boroPro moiety was present as both the D-form
and L-form while Ala and Pro were both the L-isomer.

        High pressure liquid chromatography (HPLC) can be
used to separate L-Pro-D-boroPro from L-Pro-L-boroPro. A
15   4.6 mm x 250 mm Nucleosil C18 (5μ particle) column
employing a two buffer system (Buffer A is 100% H_2O with
0.1% TFA, and buffer B is 70% CH_3CN, 30% H_2O, 0.86% TFA)
can be used to carry out the seperation. From 0 to 5 min
5% B and 95% A is used, and from 5 to 25 min 5% to 100% B
20   is used. The L,L isomer comes off first at about 7 min,
followed by the L,D isomer at about 10 min. NMR and mass
spectra analysis were consistent with both compounds
being Pro-boroPro. Rechromatography of the purified
isomers indicated that the first pass on the HPLC column
25   achieved an isomeric purity of about 99-6% for each
isomer. High pressure liquid chromatography (HPLC) can
similarly be used to be used to separate L-Ala-D-boroPro
from L-Ala-L-boroPro or to seperate the D-boroPro form of
other inhibitors from the L-boroPro form.

30   When L-Pro-L-boroPro and L-Pro-L-boroPro were used
in a DP-IV inhibition assay, the K_i for L-Pro-D-boroPro
was 3.2×10^{-11}M, while for L-Pro-D-boroPro the K_i was
6.0×10^{-8}M. The L,L-isomer constitutes a much better
inhibitor for DP-IV than the L,D-isomer. Further it is
35   preferred that all of the amino acid residues of the DP-

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IV inhibitors of the invention be the L-isomer rather
than the D-isomer.

Use

The inhibitory compounds can be administered in an
5   effective amount either alone or in combination with a
pharmaceutically acceptable carrier or diluent.

The above inhibitory compounds are useful for
treatment of a wide variety of disease; for example, an
autoimmune disease, the pathogenesis of which is
10   dependent on T cell activity. DP-IV plays a role in such
autoimmune disease and inhibition of DP-IV activity
allows regulation of the progress of the disease. Such
diseases include arthritis, rejection of transplanted
organs, as well as SLE and AIDS. When administered to
15   mammals (e.g., orally, topically, intramuscularly,
intraperitoneally, intravenously, parenterally, nasally
or by suppository), the inhibitory compounds of this
invention enhance the ability of, e.g., the immune system
of the mammal, to fight the disease.
20   Inhibitors of DP-IV can suppress IL-2 production
and thus diseases in which the production of IL-2 is
altered may be treated by use of these inhibitors. These
inhibitors can also delay catabolism of growth hormone
releasing factor, and block DP-IV activity of amoebae and
25   microbial pathogens to allow an immune system to act more
efficiently.

The inhibitory compounds or compositions can be
administered alone or in combination with one another, or
in combination with other therapeutic agents. The dosage
30   level may be between 1 - 500 mg/kg/day.

Other Embodiments

Other embodiments are within the following claims.
For example, other inhibitors can be created which mimic
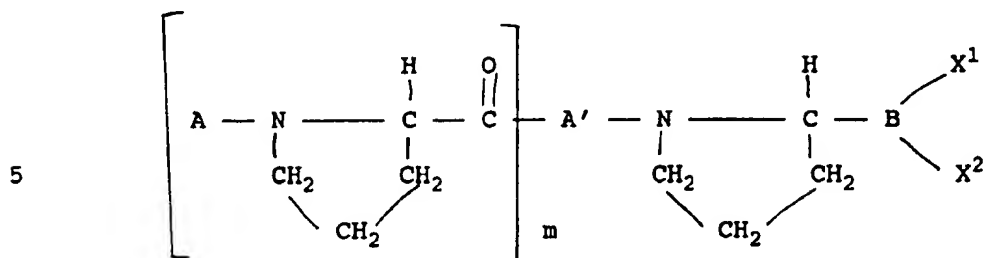the structure of Ala-boroPro. Examples of such

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inhibitors are shown in Fig. 2 and include Ala-boroPro. These inhibitors generally have a boroPro group, or its equivalent, described above in the Summary of the Invention, and a positively charged amine group. The

5   inhibitors are designed so that minimal interaction of the amine and boroPro groups occurs, and thus no cyclic structure is formed at pH 7.0. These inhibitors interact and/or bind with DP-IV, and thereby reduce the DP-IV enzymatic activity toward a normal substrate. These

10   inhibitors are synthesized by procedures well known to those of ordinary skill in this art.

Claims: - 23 -

1.   An inhibitor of DP-IV, having the structure:

$$\left[A - N \underset{\underset{\diagdown\;CH_2}{CH_2}}{\overset{H}{|}} \; \overset{H}{\underset{CH_2}{C}} - \overset{O}{\overset{||}{C}} \right]_m A' - N \underset{\underset{\diagdown\;CH_2}{CH_2}}{\overset{H}{|}} \; \overset{H}{\underset{CH_2}{C}} - B \diagup^{X^1} \diagdown_{X^2}$$

wherein m is an integer between 0 and 10, inclusive; A
and A' are L-amino acid residues such that the A in each
repeating bracketed unit can be a different amino acid
residue; the C bonded to B is in the L-configuration; the
bonds between A and N, A and C, and between A and N are
peptide bonds; and each X^1 and X^2 is, independently, a
hydroxyl group or a group capable of being hydrolysed to
a hydroxyl group at physiological pH.

2.   The inhibitor of claim 1 wherein A and A' are
independently proline or alanine residues.

3.   The inhibitor of claim 1 wherein m is 0.

4.   The inhibitor of claim 1 wherein X^1 and X^2 are
hydroxyl groups.

5.   The inhibitor of claim 1 wherein said
inhibitor is L-Ala-L-boroPro.

6.   The inhibitor of claim 1 wherein said
inhibitor is L-Pro-L-boroPro.
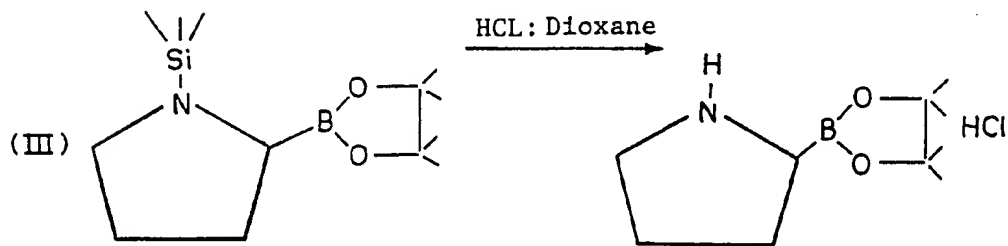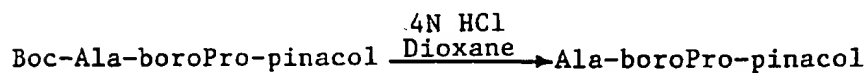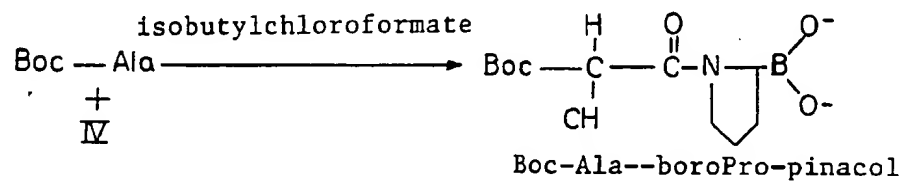
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FIG.I

(I) Br-(CH₂)₃-CH-B ⎯[(CH₃)₃ Si]₂ N-Li⁺⟶
         | -78°C - +25°C
         Cl

4-bromo-1-chlorobutyl boronate pinacol

(II) Br-(CH₂)₃-CH-B ⎯Distillation⟶
           |
           N
          Si Si
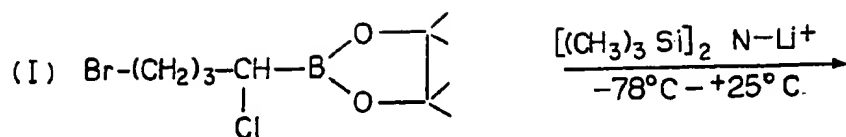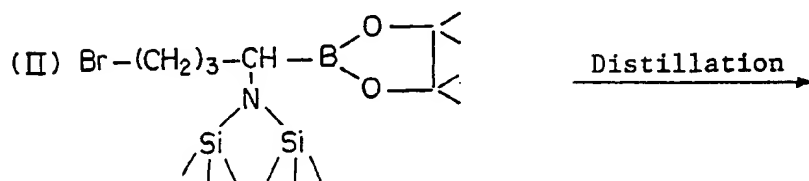
4-bromo-1[(bistrimethylsilyl) amino] butyl bornonate pinacol

(III) 1-trimethylsilyl-boroProline pinacol ⎯HCL: Dioxane⟶ (IV) boroProline-pinacol-HCL

Boc — Ala ⎯isobutylchloroformate⟶ Boc-Ala--boroPro-pinacol
    +
    IV

Boc-Ala-boroPro-pinacol ⎯4N HCl / Dioxane⟶ Ala-boroPro-pinacol

SUBSTITUTE SHEET

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FIG.2

R = H, CH₃

n=1-3

R=alkyl (C₁-C₃), H
n=1-3

n=2-4